

REMARKS

Claims 1, 3, 4, and 6-50 were pending. Claims 36-50 were withdrawn from consideration, and claims 2 and 5 were cancelled. By virtue of this response, claims 1, 4, 6, 7, 13, and 19 are amended; claims 3, 10-12, 14-17, 20-22, 25-27, and 32-35 are cancelled; and new claims 51-56 are added. Support for the amendment of claim 1 is found in the specification on, *inter alia*, page 18, lines 8-30. Claim 6 is amended be an independent claim. Support for the amendment of claim 13 is found in the specification on, *inter alia*, page 7, lines 3-5. Support for new claims 51-56 is found in the original claims 4, 7, 18, 19, 23, and 30, and in the specification on, *inter alia*, page 5, lines 24-25 and 17-20; page 7, lines 7-12, 22-23 and 28-30. Accordingly, claims 1, 4, 6-9, 13, 18, 19, 23, 24, 28-31, and 51-56 are under consideration.

The amendments are made solely to promote prosecution without prejudice or disclaimer of any previously claimed subject matter. With respect to all amendments and cancelled claims, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover has not acquiesced to any rejections and/or objections made by the Patent Office. Applicant expressly reserves the right to pursue prosecution of any presently excluded subject matter or claim embodiments in one or more future continuation and/or divisional application(s).

Applicant has carefully considered the points raised in the Advisory Action and believes that the Examiner's concerns have been addressed as described herein, thereby placing this case into condition for allowance.

Claim objections and rejections under 35 U.S.C. §112, second paragraph

The Examiner states that amendments to the claims in the previous response filed December 9, 2004 seem to overcome the objections and rejections under 35 U.S.C. §112, second paragraph. Applicant respectfully request that the Examiner withdraw the objections and rejections.

Rejections under 35 U.S.C. §112, first paragraph***Written Description***

Claims 1, 3-4, 6, 8-35 remain rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner states that neither the specification nor the art provide any information as to the level of structural conservation among all mammalian SAH hydrolase and the structural elements most likely to be conserved among all mammalian SAH hydrolase, such that one of skilled in the art can predict the structures of other mammalian SAH hydrolase with the only species known, i.e., one mouse, one human, and several rat SAH hydrolases. The Examiner further states that there is no clue as to the structural elements in all mammalian SAH hydrolases which are mostly likely to be variable and the role of this variability in SAH hydrolase activity or binding affinity. The Examiner concludes that neither the art nor the specification provide a correlation between structure and function for the entire genus of mammalian SAH hydrolase recited in the claims.

Applicant respectfully traverses this rejection.

Applicant respectfully notes that, in the interest of expediting prosecution and without acquiescence to the rejections, claim 1 is amended to recite that the mutant SAH hydrolase is derived from an SAH hydrolase encoded by a known nucleic acid sequence, and claim 6 is amended to recite that the mutant SAH hydrolase is derived from a human SAH hydrolase.

Applicant respectfully submits that the claims as amended are supported by structural features and by functional characteristics coupled with a known correlation between function and structure. Structural elements for these SAH hydrolase are provide by the sequences. One skilled in the art can mutate these genes to obtain mutant SAH hydrolase having binding affinity for Hcy, SAH or adenosine but having attenuated catalytic activity as claimed based on their homology to the rat and human amino acid residues that are directly interacting with and amino acid residues that are adjacent to amino acid residues that are directly interacting with the substrate and coenzyme.

A human SAH hydrolase sequence (SEQ ID NO:1) is provided in the specification. Applicant also respectfully submits that only one human gene encoding human SAH hydrolase has been identified and no tissue-specific isozymes have been identified. *See* Hershield et al., Science 216:739-742 (1982) (Hershield) (Exhibit 1), which is attached with the response for Examiner's convenience. As shown in Hershield, the gene encoding human SAH hydrolase is mapped on chromosome 20. (Exhibit 1 at page 741.)

In view of the above, Applicant respectfully submits that the written description requirement has been met in view of the claim amendment, and withdrawal of this rejection is respectfully requested.

Enablement

Claims 1, 3-4, 6, 8-35 remain rejected under 35 U.S.C. §112, first paragraph, for allegedly not reasonably providing enablement for (1) a method for assaying Hcy, SAH, or adenosine using any mammalian-derived mutant SAH hydrolase having the functional characteristics recited in the claims, (2) the method of (1) further comprising detecting cholesterol and/or folic acid in the sample by any means, or (3) the method of (1) further comprising detecting cholesterol and/or folic acid in a sample by any means, wherein the mutant SAH hydrolase comprises SEQ ID NO: 1 and also has the amino acid substitutions recited in claim 7 or in the specification. The Examiner states that in the instant case the genus of mammalian SAH hydrolases required is an extremely large genus and the specification fails to provide (1) the structures of all wild-type mammalian SAH hydrolases or a correlation between structure and function among all mammalian SAH hydrolases, (2) the specific amino acid residues in all wild-type mammalian SAH hydrolases which can be modified such that the required functional characteristics recited in the claims are display, and (3) the amino acid residues which can be used to substitute those found in the wild-type mammalian SAH hydrolases to obtain the mutant SAH hydrolases.

Applicant respectfully traverses this rejection.

Applicant respectfully submits that claims as amended are enabled. The sequence of each species in claim 1 as amended provided. As discussed above, the only known human SAH hydrolase sequence is also provided. One skilled in the art could apply the teachings of the specification to mutate these genes to obtain mutant SAH hydrolase having binding affinity for Hcy, SAH or adenosine but having attenuated catalytic activity as claimed based on their homology to the rat and human amino acid residues that are directly interacting with and amino acid residues that are adjacent to amino acid residues that are directly interacting with the substrate and coenzyme without undue experimentation.

Applicant disagrees with the Examiner that the specification does not reasonably provide enablement for the method further comprising detecting cholesterol and/or folic acid in the sample by any means. However, in the interest of expediting prosecution and without acquiescence to the rejections, Applicant has cancelled claim 35. Thus, this rejection is rendered moot.

In view of the above, Applicant respectfully submits that pending claims are enabled. Applicant respectfully requests reconsideration and withdrawal of the rejections under 35 U.S.C. §112, first paragraph.

Rejections Under Nonstatutory Double Patenting

The Examiner maintains the rejection under the judicially created doctrine of obviousness-type double patenting to claims 1-3, 6, 8-9, 18-19, 23-24, 30-34 for alleged as being unpatentable over claims 1-3, 6-14, and 16 of U.S. Patent No. 6376210.

Applicant reiterates that this issue will be addressed when other rejections are withdrawn.

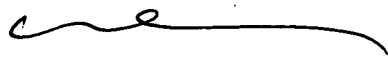
CONCLUSION

Applicant believes that all issues raised in the Office Action have been properly addressed in this response. Accordingly, reconsideration and allowance of the pending claims is respectfully requested. If the Examiner feels that a telephone interview would serve to facilitate resolution of any outstanding issues, the Examiner is encouraged to contact Applicant's representative at the telephone number below.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 466992000221.

Dated: May 9, 2005

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 22. α -Aminoisobutyric acid and norepinephrine are actively transported into cells of the central nervous system by a Na^+ -dependent process but the amino acid is not taken up by the storage vesicles and is not coreleased with norepinephrine upon electrical stimulation (F. Orrego, J. Jankelevich, L. Ceruti, E. Ferrera, *Nature (London)* 251, 55 (1974); O. Vargas and F. Orrego, *J. Neurochem.* 26, 31 (1976); F. Orrego and R. Miranda, *ibid.*, p. 1033). Therefore, α -[methyl- ^3H]aminoisobutyric acid (New England Nuclear) was used as a model for an extravesicular compartment marker.
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- * A.J.D. is a visiting scientist on leave from the Universidad Catolica de Chile, Santiago, Chile.

24 February 1982

The Human Genes for S-Adenosylhomocysteine Hydrolase and Adenosine Deaminase Are Syntenic on Chromosome 20

Abstract. Human-Chinese hamster cell hybrids and a monoclonal antibody to human S-adenosylhomocysteine hydrolase were used to identify chromosome 20 as the location of the human gene for this enzyme. The gene for adenosine deaminase had previously been mapped to this chromosome. The activity of S-adenosylhomocysteine hydrolase is dependent in vivo on that of adenosine deaminase, since the substrates for the deaminase, adenosine and deoxyadenosine, respectively, inhibit and inactivate S-adenosylhomocysteine hydrolase in genetic or drug-induced adenosine deaminase deficiency. This functional dependence and the likelihood that S-adenosylhomocysteine hydrolase, a eukaryotic enzyme, arose later than adenosine deaminase, which occurs in prokaryotes as well as eukaryotes, suggest that the occurrence of their genes on the same chromosome may have evolutionary significance. In addition, the unusual capacity of S-adenosylhomocysteine hydrolase to form stable complexes with adenosine and its cofactor, nicotinamide adenine dinucleotide, suggest that evolution of its gene may have involved recombination of a portion of the adenosine deaminase gene with an adenine nucleotide domain-coding sequence of another preexisting gene.

In genetic deficiency of adenosine deaminase (ADA; E.C. 3.5.4.4) (1), accumulation of adenosine and 2'-deoxyadenosine results in profound depletion of lymphoid tissues and severe combined immunodeficiency disease. Investigation into the biochemical effects of these purine nucleosides has led to recognition that the activity of a second enzyme involved in the metabolism of adenosine, S-adenosylhomocysteine (AdoHcy) hydrolase (E.C. 3.3.1.1), is dependent in vivo on that of ADA. S-Adenosylhomocysteine hydrolase catalyzes the reversible hydrolysis of AdoHcy to adenosine and L-homocysteine (2). This reaction is important because AdoHcy is a potent inhibitor, as well as a product, of S-adenosylmethionine-dependent transmethylation, a step that occurs in the biosynthesis, modification, or regulation of the function of nucleic acids, proteins, phospholipids, and biogenic amines (3) (see Fig. 1). Cleavage of AdoHcy is the only source of homocysteine, which is used

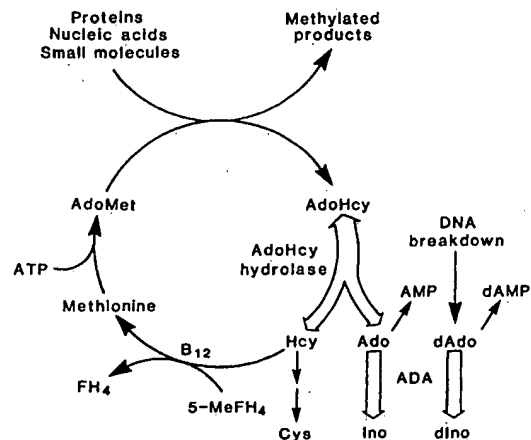
in the synthesis of the essential amino acid methionine in a reaction that also produces tetrahydrofolate, a cofactor for several enzymes participating in the synthesis of nucleic acid precursors.

Two mechanisms account for the dependence of the activity of AdoHcy hydrolase on that of ADA. First, because the equilibrium constant for AdoHcy hydrolysis is highly unfavorable (2), further metabolism of adenosine or homocysteine is required to promote AdoHcy cleavage and prevent its accumulation. In cultured lymphoid cells treated with ADA inhibitors, AdoHcy accumulation, causing inhibition of transmethylation reactions, is largely responsible for the toxic effects of adenosine in the concentration range 5 to 50 μM (4, 5). In addition to the inhibitory effect of adenosine, we have shown that deoxyadenosine is a "suicide-like" substrate analog that causes the inactivation of purified AdoHcy hydrolase (6), and of AdoHcy hydrolase in ADA-inhibited cultured cells (7). This effect of 2'-deoxyadenosine results in severe deficiency of AdoHcy hydrolase activity in the erythrocytes of ADA-deficient children (8), and in the erythrocytes and lymphoblasts of patients with lymphocytic leukemias when they are treated with the drug 2'-deoxycytosine, a potent inhibitor of ADA that causes deoxyadenosine accumulation in vivo (9, 10).

We now report that the genes for ADA and AdoHcy hydrolase are located on the same human chromosome. We mapped the location of AdoHcy hydrolase by using a mouse monoclonal antibody directed against human placental AdoHcy hydrolase (11) to develop an immunoprecipitation method for detecting human AdoHcy hydrolase in human-hamster hybrid cell lines that contain different subsets of human chromosomes. Somatic cell hybrids and monoclonal antibodies have recently been used to map the genes for human liver and muscle isozymes of 6-phosphofructokinase (12).

The monoclonal antibody designated

Fig. 1. Metabolic functions of AdoHcy hydrolase and ADA. Abbreviations: AdoMet, S-adenosylmethionine; FH₄, tetrahydrofolate; 5-MeFH₄, 5-methyltetrahydrofolate; B₁₂, vitamin B₁₂; Hcy, homocysteine; Cys, cysteine; Ado, adenosine; Ino, inosine; dAdo, 2'-deoxyadenosine; dIno, 2'-deoxyinosine; AMP, adenosine monophosphate; dAMP, 2'-deoxyadenosine monophosphate; ADA, adenosine deaminase; and AdoHcy, S-adenosylhomocysteine.



Hybrid	AdoHcy hydrolase activity		Presence of human AdoHcy hydrolase	Presence of human ADA
	Total in incubation (nmole/hour)	In pellet (%)		
X-7A	62.2	29.8	+	+
XII-2D	82.0	0.1	—	—
XII-12B	143.7	0.3	—	—
XIII-1A	116.7	21.5	+	+
XIII-1B	76.1	27.8	+	+
XIII-3A	178.9	1.4	—	—
XIII-4D-1c	60.0	0.5	—	—
XV-15A-4a	70.8	16.2	+	+
XV-16B	114.3	20.7	+	N.D.
XV-16B ^{aza}	61.2	13.2	+	+
XV-18A-8b	61.9	30.3	+	N.D.
XV-18B-6a	49.3	39.6	+	+
XV-18B-7a	83.8	41.4	+	+
XXI-51B	95.3	8.9	+	+

Antibody (µg)	Human Supernatant (○)	Human Supernatant (●)	Human Pellet (○)	Human Pellet (●)	Hamster Supernatant (△)	Hamster Supernatant (▲)	Hamster Pellet (△)	Hamster Pellet (▲)
0	5	55	5	5	50	55	5	5
1	15	18	15	15	50	52	5	5
2	24	2	24	2	52	55	5	5
6	24	2	24	2	42	42	5	5
10	24	2	24	2	27	27	5	5

Table 2. Assignment of gene for AdoHcy hydrolase to chromosome 20 by concordant segregation in a panel of Chinese hamster-human hybrids.

Hybrid*	Human AdoHcy hydrolase	Human chromosome (mean copy number per cell)																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
X-7A	+	1.0	0.5	1.5	1.0	0.9	1.4	0.4	0.9	0.9	0.7	1.2	1.4	1.3	0.5	1.0	0.4	0.3	1.1	0.5	0.9	0.8	0.9	0.7	
XII-2D	-	0.8	0.4	1.5	1.0		1.1		1.0	0.5			0.8	1.0	0.9	1.3	0.9		1.2	0.9		1.0	1.0	1.0	
XII-12B	-	0.8		1.0	1.0		1.0		0.8			0.8			0.9	0.9	0.9		1.6	1.0		0.9	0.8		
XIII-1A	+								0.7	0.9	1.0				0.2	0.9	0.6		0.5		1.0	0.9	0.8		
XIII-1B	+																								
XIII-3A	+																								
XIII-4D-1c	-							0.5			0.1		0.8	0.8	0.4†		0.5				0.6		0.3	0.9	
XV-15A-4a	-			0.5			0.9																		
XV-16B	+	0.2‡	0.6	0.8	0.8		0.2‡	0.7	0.9	0.4	1.2	1.0	0.5	1.0	1.0		1.2	0.5	0.5		0.8	0.3	0.8	0.9	
XV-16B aza	+	0.9§	0.7	1.0	1.0	0.9	0.9	1.0	0.8	0.8	1.0	0.8	1.0	1.0	1.0	0.9	1.0	1.0	1.7	0.9	0.7	1.0	1.0	1.0	
XV-18A-8b	+	1.0§	0.7	0.9	0.9	1.0	0.8	0.9	0.6				0.6	1.6	0.7				0.7	0.8	0.6	0.9	0.8		
XV-18B-6a	+	0.8	0.9	1.4	0.9	0.9	0.9	1.0		0.9	0.7	1.0	1.9	0.7	0.9	1.5	1.0	0.7	0.8	0.5	1.4	0.7			
XV-18B-7a	+	1.0§	0.7	1.1		0.9	1.0§		0.8	0.9	0.9	0.9	0.9	0.8	0.9	0.8	0.8	0.8	0.8	0.7	0.9	0.9	1.2	0.8	
XXI-51B	+			0.9	0.7	0.6		1.3	0.8	0.2	0.6	0.7	0.9	0.7	0.7	0.8	0.8	0.2	0.7	0.7	0.6		0.9		
Ratio discordant		5/8	4/13	5/13	5/13	3/13	7/11	6/13	5/13	6/13	5/12	4/13	6/13	3/12	5/13	5/13	4/13	5/13	4/13	5/13	0/13	4/13	4/13	5/13	
Percent discordant		63	31	38	38	23	64	46	38	46	42	31	46	46	25	38	31	38	31	38	0	31	31	38	
*The derivation of the different series of hybrids has been summarized in (30). †der(X), t(X;14)(p22;q21). ‡der(1), t(1;6)(p32;p21). §der(6), t(1;6)(p32;p21). Hybrids in which only parts of relevant chromosomes were present, or in which they were present in less than 15 percent of cells, were excluded from calculations of discordance.																									

*The derivation of the different series of hybrids has been summarized in (30). $t_{\text{der}}(X)$, $t(X;14)p22;q21$, were present, or in which they were present in less than 15 percent of cells, were excluded from calculations of $t_{\text{der}}(X)$.

B2A3, in the presence of heat-killed, Formalin-fixed staphylococcus (13), was used to precipitate the AdoHcy hydrolase activity from an extract of human lymphoblastoid cells (Fig. 2). About 60 to 80 percent of the activity removed by centrifugation was recovered in the staphylococcal pellet. Approximately ten times more B2A3 antibody was required to precipitate an equivalent amount of AdoHcy hydrolase activity from an extract of Chinese hamster cells. However, a somewhat lower percentage of hamster activity was recovered in the pellet, probably reflecting release of the more weakly bound hamster AdoHcy hydrolase in the process of washing the pellet before assay. Neither the staphylococcal suspension nor the B2A3 antibody alone inhibited either human or hamster AdoHcy hydrolase activity in solution (not shown). With the proper concentration of antibody (indicated by the arrow in Fig. 2), the presence of the human enzyme is easily determined in mixtures containing various amounts of human and hamster activities by assaying for activity in washed staphylococcal pellets (not shown).

For mapping studies, we used a series of hybrid cell lines obtained from fusions of the Chinese hamster lung fibroblast cell line V-79 (similar to line 743) with either human fibroblasts, or human blood leukocytes. The 14 hybrid clones examined were derived from four different human donors. Each hybrid clone was karyotyped at a passage close to the one at which cells were harvested for enzyme studies. The frequency of occurrence of each human chromosome was determined by analysis of 10 to 35 trypsin-Giemsa-banded metaphase spreads. Extracts of the hybrid clones were analyzed for immunoprecipitable AdoHcy hydrolase with the B2A3 monoclonal antibody (Table 1), and the results were correlated with the human chromosome contents of the hybrids (Table 2).

Clone X-7A was used as a positive hybrid control, since it contains at least one copy of all human chromosomes. Roughly 30 percent of the AdoHcy hydrolase activity in the extract of this hybrid was precipitated by antibody to human AdoHcy hydrolase. In the 13 hybrids that were informative for mapping, human AdoHcy hydrolase expression segregated concordantly with chromosome 20. For all other autosomes and the X chromosome, the fraction of discordant clones was 23 percent or greater (Table 2). The assignment of the gene for AdoHcy hydrolase (for which we suggest the abbreviation *AHCY*) to chromosome 20 was further confirmed by demonstrating concordant segregation with

ADA [cellulose acetate electrophoresis (14) being used to identify the presence of the human *ADA* isozyme in extracts of the hybrid clones] (Table 1). The gene for *ADA* has been mapped to the distal long arm of chromosome 20 (15).

Information on the distance between *ADA* and *AHCY* is not yet available. Their synteny could be due to chance alone. The probability of a gene occurring on chromosome 20 is 1 in 22 if only the haploid number of human autosomes is considered, but since chromosome 20 contains only about 2.4 percent of the total haploid autosomal length (16), the chance of any locus occurring on chromosome 20 is about 1 in 40.

There is abundant evidence of the conservation during evolution of amino acid sequences in the enzymes that catalyze the same metabolic reactions in prokaryotes and eukaryotes (17). However, the organization into linkage groups of the genes for enzymes that function sequentially in metabolic pathways, a frequent occurrence in prokaryotic chromosomes, is uncommon in eukaryotes. Apparently gene linkage has usually not conferred much additional selective advantage over conservation of the DNA coding sequences themselves. There are examples of probable gene linkages for the multifunctional proteins that contain the first three (18–20) and the last two enzyme activities of pyrimidine biosynthesis (21, 22). The genes coding for the third and sixth enzymes in the pathway of de novo purine nucleotide synthesis have been reported to map to human chromosome 21 (23). In the latter instance, there appears to be coordinate expression of these genes, which code for separate polypeptide chains. Two possibilities suggest themselves as factors that might be responsible for non-random occurrence of the genes for *ADA* and AdoHcy hydrolase on the same chromosome.

First, there is evidence that in the absence of *ADA*, its substrates, adenosine and deoxyadenosine, respectively, inhibit (2, 4, 5) and inactivate (6–8) intracellular AdoHcy hydrolase. Adenosine deaminase appears to have evolved before AdoHcy hydrolase, since *ADA* occurs in prokaryotes as well as eukaryotes, but AdoHcy hydrolase occurs only in the latter (3). Evolution of a catalytically efficient AdoHcy hydrolase, which presumably required a long time, may have first occurred in a cell in which the simultaneous presence of *ADA* activity was ensured by linkage of the *ADA* and evolving AdoHcy hydrolase genes.

The second, but not necessarily independent, possibility has to do with some unusual properties of AdoHcy hydro-

lase, which suggest that its evolution in eukaryotes may have involved the fusion of specialized domain-coding regions of preexisting genes for other proteins. *S*-Adenosylhomocysteine hydrolase is a tetramer with subunits of molecular weight 46,000 to 50,000 that contain one molecule of tightly, but not covalently, bound nicotinamide adenine dinucleotide (NAD^+) per subunit (24–26); no tissue-specific isozymes have been reported. In addition to its catalytic function, we have shown (26) that AdoHcy hydrolase can form a stable complex with adenosine (dissociation constant, $2 \times 10^{-7}M$ to $5 \times 10^{-7}M$), and have identified it as the cytoplasmic high-affinity "cyclic AMP-adenosine binding protein" (cyclic AMP is adenosine 3', 5'-monophosphate) that had been isolated from various sources (27). The significance of this capacity of AdoHcy hydrolase to bind adenosine and cyclic AMP remains unclear. It is possible that a region of the AdoHcy hydrolase gene arose by tandem reduplication of, or recombination with, a portion of the *ADA* gene coding for an adenosine binding site, but lacking the nucleotide sequences that encode the catalytic (deamination) site of *ADA*. This region may have become combined with a DNA sequence encoding a primitive NAD^+ -binding domain, and by further mutation and selection evolved into the AdoHcy hydrolase gene.

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31. Supported by NIH grant AM 20902 and research career development award AM 00424 (to M.S.H.) and by NIH grant GM 26105 (to U.F.). We thank J. E. Fetter and J. E. Misenheimer for technical assistance. We thank G. Eisenbarth, Duke University, for the mouse myeloma cell line.

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24 December 1981

Adenosine Triphosphate Synthesis Coupled to K⁺ Influx in Mitochondria

Abstract. *The influx of K⁺ into swollen mitochondria in the presence of valinomycin results in the synthesis of adenosine triphosphate in which approximately one H⁺ disappears per adenosine triphosphate synthesized. The synthesis is blocked by atractyloside but is insensitive to oligomycin and relatively insensitive to uncouplers.*

The reversibility of some cation transport pumps has been demonstrated in recent years (1-6). A net synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i) occurs when the systems are run in reverse. In mitochondria, ATP synthesis is coupled to K⁺ efflux in the presence of valinomycin (1, 2, 6). Cockrell and Pressman (6) reported 1.8 ± 0.7 μmole of ATP and 3.2 ± 0.4 μmole of ATP synthesized per gram of protein, respectively, in the absence and in the presence of a glucose-hexokinase trap. Rossi and Azzone (2), using the glucose-hexokinase trap, report values as high as 15 to 20 μmole per gram of protein, probably because of their pre-

loading of the mitochondria with K⁺. An energy-dependent net efflux of K⁺ has been demonstrated in swollen mitochondria (7).

In view of the reversibility of ion transport phenomena, we tested the possibility of synthesizing ATP in osmotically swollen mitochondria under conditions favoring a passive influx of K⁺ in the absence of oxidative metabolism. Our results demonstrate the synthesis of 3 to 7 μmole of ATP per gram of protein per minute, and as much as 14.8 μmole per gram of protein per minute in the presence of a glucose-hexokinase trap and 150 mM KCl in the medium. The total ATP synthesized was as high as 16 μmole per gram of protein. The swollen

mitochondria had been somewhat depleted of their internal K⁺, which in two preparations corresponded to 27 ± 8 and 46 ± 5 μmole of K⁺ per gram of protein, compared to 180 ± 40 found in fresh mitochondria (8). After valinomycin treatment, the internal K⁺ increased in proportion to the external K⁺ (not shown). In these experiments mitochondria were rapidly centrifuged through a silicon layer into a HClO₄ solution (9). The K⁺ concentrations, determined by atomic absorption, were calculated after correction for the [carboxyl-¹⁴C]carboxyldextran space (10). These experiments show that the ATP synthesis coincides with a K⁺ influx. The ATP formed probably does not involve Ca²⁺ exchanges, since the same results were obtained after washing the mitochondria twice with 1 mM EGTA.

When mitochondria were suspended in a medium containing a high concentration of KCl and valinomycin at pH 7.8, P_i disappeared from the suspension (Table 1 and Fig. 1A) [the medium and the mitochondria were analyzed together (10)]. This disappearance approximately matched the ATP synthesized, as determined with the luciferin-luciferase reaction (Table 1). These results indicate that the observed P_i disappearance represents ATP production from ADP and P_i. This conclusion is supported by the inhibition of the P_i disappearance by atractyloside (Table 2), which blocks adenine nucleotide transport (11), and by the apparent stoichiometric uptake of H⁺ accompanying the synthesis (see below). The effectiveness of the atractyloside also indicates that the orientation of the mitochondrial membrane (the sidedness) remained unchanged after the preparatory procedure.

In the presence of antimycin A and rotenone, no respiration was observed during the usual time course of the experiment (Table 3). In addition, the presence of 2 mM KCN had no effect on the phosphorylation in the presence of valinomycin and high KCl (experiment 2 in Table 3 and experiments 3b and 3c in Table 4). Therefore, the phosphorylation was not the result of residual oxidative phosphorylation.

The metabolically blocked preparation continuously produced H⁺ (Fig. 1A), as previously observed (12). After the addition of valinomycin and a delay of about 1 minute, the disappearance of P_i took place and was matched by the disappearance of H⁺, typically in approximately one-to-one stoichiometry (see legend to Fig. 1A). This stoichiometry is expected, at least approximately, from the reaction ADP + P_i + H⁺ → ATP (13). General-

Table 1. Comparison of phosphate disappearance and ATP production. Mitochondria were isolated (18), then washed twice in cold 0.15M sucrose and 10 mM tris, pH 7.4. Disappearance of P_i from the whole suspension was measured colorimetrically (19) with an automated system (Autotechnicon) or conventionally. Adenosine triphosphate was estimated with luciferin-luciferase (DuPont) (3 mg in 0.1M MgSO₄ and 0.1M tris, pH 7.6) in a photometer (Aminco-Chem-Glow) with an integrator, after removal of the mitochondria. Background ATP was estimated with time in parallel on portions withdrawn just before addition of valinomycin. Oxygen was measured with a Clark electrode (Yellow Springs, model 53). The incubation medium was 0.15M KCl and 10 mM tris, pH 7.8, containing 0.33 mM ADP and 0.5 mM Na₂HPO₄, and maintained at 25°C. Rotenone and antimycin A were present at a concentration of 0.2 μg/ml. Phosphorylation and ATP levels were monitored in parallel. In experiments 1 and 2, 3 mM glycylglycine replaced the tris buffer. The mitochondria were present at 1.3 to 1.8 mg of protein per milliliter, and the concentration of valinomycin was 0.33 to 0.66 μg/ml. Generally, the optimal valinomycin concentration was determined at the onset of the experiment. In all tables and the figure, results are expressed as means ± standard deviation of at least four determinations, except where noted.

Experiment	P _i (nmole/min-mg)	ATP (nmole/min-mg)
1	5.0 ± 1.7	3.5 ± 2.8
2	3.6 ± 0.6	2.4 ± 1.6
3	7.0 ± 2.3	13.6 ± 1.8